Electronic Supplementary Information

Paclitaxel nanoparticle awakens immune system to fight against cancer

Wei Tang,[#a] Jingbo Yang,[#b] Yue Yuan,[a] Zhibin Zhao,[b] Zhexiong Lian*[b] & Gaolin Liang*[a]

[a]CAS Key Laboratory of Soft Matter Chemistry, Department of Chemistry, University of Science and Technology of China, 96 Jinzhai Road, Hefei, Anhui 230026, China

[b]Liver Immunology Laboratory, Institute of Immunology and School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, China

[#]These authors contributed equally to this work

Correspondence and requests for materials should be addressed to e-mail: e-mail: zxlian1@ustc.edu.cn (Z. L.) and e-mail: gliang@ustc.edu.cn (G. L.).

Contents

1. General methods

All the starting materials were obtained from Adamas or Sangon Biotech. Commercially available reagents were used without further purification, unless noted otherwise. All chemicals were reagent grade or better. Paclitaxel (**PTX**) was obtained from Baomanbio (Shanghai, China). HPLC analyses were performed on an Agilent 1200 HPLC system equipped with a G1322A pump and in-line diode array UV detector using an Agilent Zorbax 300SB-C18 RP column with CH_3CN (0.1% of trifluoroacetic acid (TFA)) and water (0.1% of TFA) as the eluent. UV-vis absorption spectra were recorded by a Pekin-Elmer lambda 25 spectrophotometer. Dynamic light scattering (DLS) was measured on a Zeta Sizer Nano Series (Malvern Instruments). Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, Hycolon) supplemented with 10 % fetal bovine serum at 37 °C, 5% CO_2, and humid atmosphere.

Mice

C57BL/6 (B6) mice were purchased from the Beijing Vital River Laboratory Animal Technology Co. Ltd. All mice were housed in a specific pathogen-free and controlled environment (22 °C, 55% humidity, and 12-h day/night rhythm) and care provided according to the regulations of animal care at University of Science and Technology of China (Hefei, Anhui, China). All animal experiments were performed using 6-8 week old mice.

Bone Marrow-Derived Macrophage Culture

BMDMs were derived from C56BL/6 mice as described.[1] Mice were purchased from the Experimental Animal Center, Chinese Academy of Science (Shanghai, China). All animal experiments were approved by the Ethics Committee of University of Science and Technology of China. Briefly, tibia and femoral bone marrow cells were isolated and cultured in DMEM complemented with 10% FBS in the presence of culture supernatants of L929 mouse fibroblasts. Before different stimulations, L929 culture supernatants were replaced with DMEM and starved for 4 hours (M0). For M1 polarization, 100 ng/mL LPS and 20 ng/mL IFN-γ were added to BMDMs for

24 h. For M2 polarization, 10 ng/ml IL-4 (PeproTech Inc., Rocky Hill, USA) and 10 ng/ml IL-13 (PeproTech) were added to BMDMs for 24 h.

Co-incubating experiments

BMDMs were divided into 3 groups: M0, M1, and M2 group. M0 cells were cultured freely without stimulation, M1 cells were cultured with 100 ng/mL LPS and 20 ng/mL IFN-γ, and M2 cells were cultured with 10 ng/mL IL-4 and 10 ng/mL IL-13 24 h before the addition of chemicals (*i.e.*, **1-NP**, **PTX**, or **1-Pro**) to a final concentration of 200 nM. After 48 h co-incubation, cells were digested with 5 mM EDTA and labeled with fluorescent antibodies and then analysed with a FACS Verse flow cytometer (BD Bioscience, San Jose, CA, USA).

Establishment of Melanoma Tumor Model

The mouse melanoma cell line B16F10 was kindly gifted from Prof. Zhigang Tian (University of Science and Technology of China) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.[2] About 2 × 105 B16F10 tumor cells were suspended in PBS and subcutaneously injected into the shaved flank skin of the aged-match mice. Tumors were measured everyday with digital calipers, and tumor volumes were calculated by 0.52 × length × width × width. After mice were killed, tumors and main immune organs were excised, minced and treated with RBC lysis buffer to lyse erythrocytes, made into single-cell suspensions for lymphocytes analysis.

Flow cytometry

Cell suspensions were incubated with anti-mouse CD16/32 (Biolegend, San Diego, CA) to block the Fc receptor before cell surface staining. All flow antibodies, unless otherwise noted, were purchased from Biolegend. To identify phenotypes and functional indexes of BMDMs, cells were

stained with FITC-MHC-II (M5/114.15.2), PE-CD206 (C068C2), PerCP-Cy5.5-CD11c (N418), PE/Cy7-CD80 (16-10A1), APC-F4/80 (CD8A), APC-Cy7-CD86 (GL1), V500-CD11b (M1/70). Normal IgG isotype controls (Biolegend) were used as controls. Flow data were acquired on a flow cytometer FACSVerse (BD Bioscience). Data were analyzed with Flow Jo software (Tree Star, Inc., Ashland, OR).

RT-PCR

Total RNA from BMDM was extracted with RNAiso Plus (Takara, Dalian, China), and cDNA synthesized with the PrimeScript RT reagent Kit (Takara, Dalian, China). Quantitative PCR was performed using a SYBR Premix Ex TaqTM II (Takara, Dalian, China). Data were collected on an ABI StepOne real-time PCR system (Applied Biosystems, Carlsbad, CA). The PCR primers used in this study are listed in Table S1. The expression levels of target genes were normalized to the housekeeping gene Gapdh (ΔCt), and the results were calculated with $2^{-\Delta\Delta}$Ctt method.

Cytometric bead array assay

The levels of interferon g (IFN-γ) and tumor necrosis factor a (TNF-α) from the serums of tumor-bearing mice were measured simultaneously with a cytometric bead array (CBA) kit (Mouse Inflammation Kit, BD Biosciences), using a FACSVerse flow cytometer with CBA software (BD Biosciences). Data analyzed with FlowJo software (Tree Star, Inc, Ashland, USA).

Alanine aminotransferase measurement

Alanine aminotransferase (ALT) measurement in mouse serum follows the standard experimental protocol as below: 10 μL of each sample or standard (in duplicate) was added to the microplate well. Then 50 μL of ALT reagent solution was added to each well. The wells were covered with the adhesive film and incubate at 37°C for 30 min. Then the adhesive film was carefully removed and 50

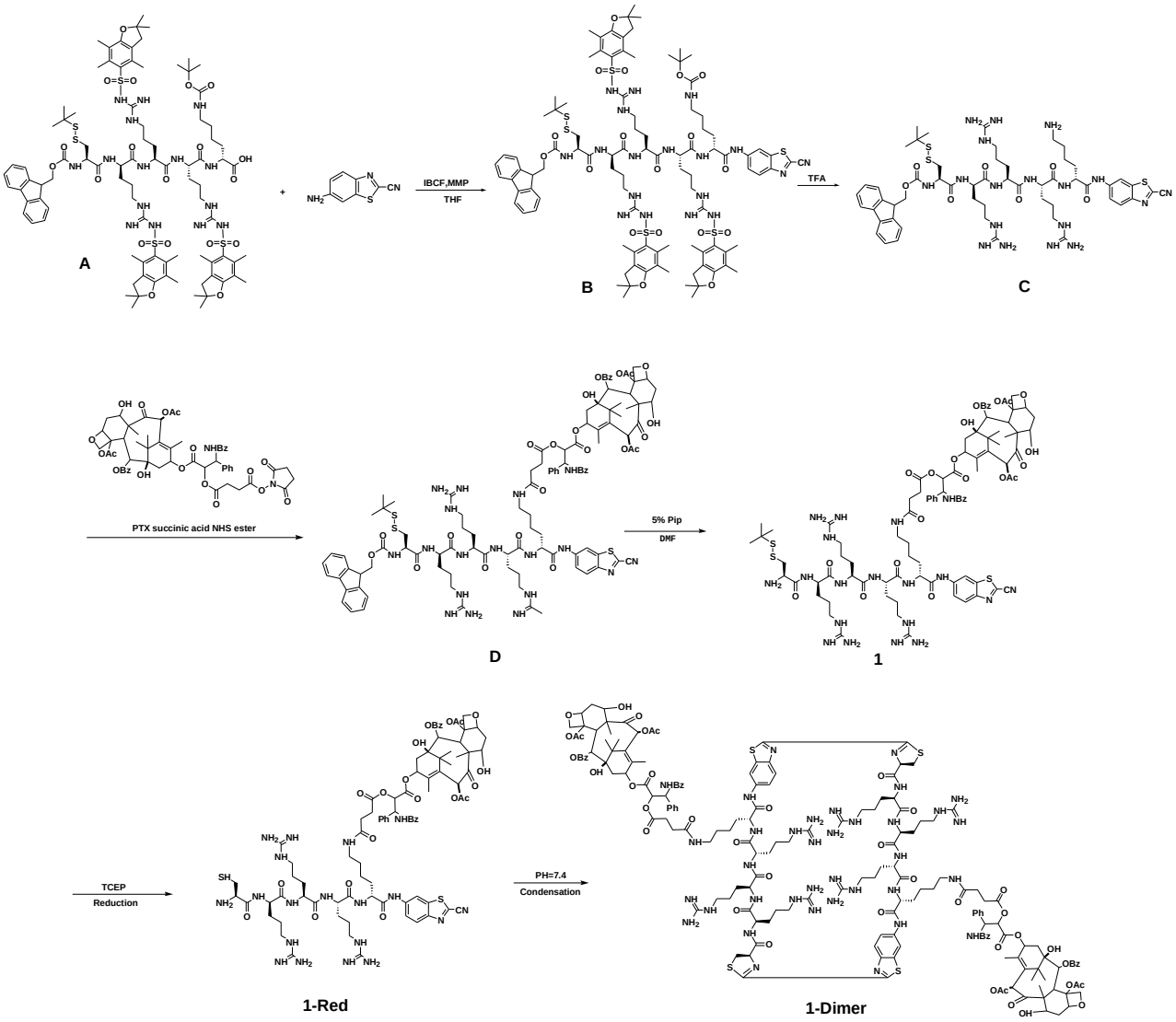
μL DNPH color solution was added to each well. The second film to was used to re-cover the wells and wells were incubate for 10 min at 37°C. The adhesive film was then removed and each well was added with 200 μL 0.5 M NaOH. 510 nm absorbance of the wells was read in plate reader. A standard curve can be constructed using the serially-diluted standards by plotting the average absorbance for each oxaloacetate standard against its concentration in U/L. Unknowns were calculated from standard curve.

2. Synthesis and Characterizations of 1 and 1-NP

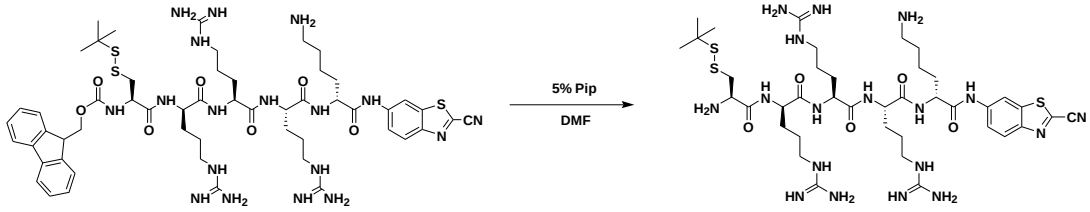
2-cyano-6-aminobenzothiazole (CBT) was synthesized following the literature method (White, E. H., Worther, H., Seliger, H. H., McElroy, W. D. Amino analogs of firefly luciferin and biological activity thereof. *J. Am. Chem. Soc.* **1966**, *88*, 2015-2019).

Preparation of ***Ac-Cys(StBu)-Arg-Arg-Arg-Lys(PTX)-CBT*** (**1**):

Scheme S1.The synthetic route for **1**.



Scheme S2. The synthetic route for **1-Pro**.



C **1-Pro**

*Synthesis of **B***: Compound **A** was synthesized with solid phase peptide synthesis (SPPS). Isobutyl chloroformate (IBCF, 87.5μL, 0.625mmol) was added to a mixture of compound **A** (450 mg, 0.24 mmol) and 4-methylmorpholine (MMP, 87.5 μL, 0.62 mmol) in THF (10.0 mL) at 0 °C under N_2.

The reaction mixture was stirred for 40 m. The solution of 2-cyano-6-aminobenzothiazole (CBT, 52 mg, 0.3 mmol) and IBCF (45 μL, 0.375 mmol) was added to the reaction mixture and further stirred for 1 h at 0 °C. Then the mixture was stirred overnight at room temperature. Compound **B** (258 mg, yield: 53%) was purified with HPLC using water-methanol added with 0.1% TFA as the eluent (from 15:85 to 0:100). MS: calculated for **B** [(M+2H$^+$)/2]: 1021.42; obsvd. ESI-MS: *m*/*z* 1021.82 (Figure S1).

*Synthesis of **C***: The Boc and Pbf protecting groups of compound **B** were removed with dichloromethane (DCM, 1 mL) and triisopropylsilane (TIPS, 200 μL) in TFA (19 mL) for 3 h. Compound **C** (236 mg, yield: 89 %) was obtained after HPLC purification using water-methanol added with 0.1% TFA as the eluent (from 5:5 to 5:95). MS: calculated for **C** [(M+2H$^+$)/2]: 593.28; [(M+TFA+2H$^+$)/2]: 650.275; obsvd. ESI-MS [(M+2H$^+$)/2]: *m*/*z* 593.275; [(M+TFA+2H$^+$)/2]: *m*/*z* 649.98 (Figure S2).

*Synthesis of **D***: **PTX** succinic acid NHS ester was synthesized following the literature method (Gao, Y., Kuang, Y., Guo, Z. F., Guo, Z. H., Krauss, I. J., Xu, B. Enzyme-Instructed Molecular Self-assembly Confers Nanofibers and a Supramolecular Hydrogel of Taxol Derivative. *J. Am. Chem. Soc.* **2009**, *131*, 13576–13577). The **PTX** succinic acid NHS ester (252 mg, 0.24 mmol) were dissolved in 10 mL DMF, then Compound **C** (236 mg, 0.2 mmol) and N,N-Diisopropylethylamine (DIPEA, 84 μL, 0.2 mmol) was added into the mixture and further stirred over night at RT to yield compound **D**. Compound **D** (300 mg, yield: 71%) was purified with HPLC using water-acetonitrile added with 0.1% TFA as the eluent (from 8:2 to 2:8). MS: calculated for **D** [(M+3H$^+$)/3]: 707.3; [(M+TFA+2H$^+$)/2]: 1117.545; obsvd. ESI-MS [(M+3H$^+$)/3]: *m*/*z* 707.96; [(M+TFA+2H$^+$)/2]: *m*/*z* 1118.36 (Figure S3).

Synthesis of ***1***: The Fmoc protecting groups of compound **D** were removed with Dimethyl Formamide (DMF, 4 mL) and Piperidine (Pip, 400 μL) for 5 m. **1** (144 mg, yield: 54%) was obtained after HPLC purification using water-methanol added with 0.1% TFA as the eluent (from 8:2 to 2:8). MS: calculated for **1** [(M+TFA+H_2O+2H^+)/2]: 1016.41072; [(M+H_2O+3H^+)/3]: 639.94547; obsvd. HR-ESI/MS [(M+TFA+H_2O+2H^+)/2]: *m/z* 1016.41236; [(M+H_2O+3H^+)/3]: *m/z* 639.94579 (Figure S4). ^{1}H NMR of **1** (d_6-DMSO, 300 MHz, Figure S5) δ (ppm): 8.69 (s, 1 H), 8.36 (s, 2 H), 8.21 (d, *J* = 9.0 Hz, 2 H), 7.94 (d, *J* = 7.3 Hz, 2 H), 7.87 (d, *J* = 7.6 Hz, 2 H), 7.79 (s, 1 H), 7.76 (s, 1 H), 7.61 (d, *J* = 6.1 Hz, 2 H), 7.55 – 7.45 (m, 6 H), 7.39 (s, 2 H), 7.27 (s, 2 H), 4.37 (s, 1 H), 4.27 (s, 3 H), 4.06 (s, 1 H), 3.09 (d, *J* = 8.6 Hz, 9 H), 2.95 (s, 3 H), 2.89 (s, 2 H), 2.73 (s, 2 H), 2.34 (d, *J* = 7.3 Hz, 2 H), 2.17 (d, *J* = 3.4 Hz, 2 H), 2.07 (d, *J* = 4.9 Hz, 6 H), 1.75 (d, *J* = 5.3 Hz, 3 H), 1.65 (d, *J* = 12.8 Hz, 6 H), 1.52 (d, *J* = 8.5 Hz, 9 H), 1.36 (s, 3 H), 1.29 (s, 9 H), 1.25 (s, 3 H), 1.19 (d, *J* = 7.6 Hz, 3 H), 0.99 (t, *J* = 12.4 Hz, 3 H), 0.91 – 0.78 (m, 6 H).

3. Supporting Figures and Tables

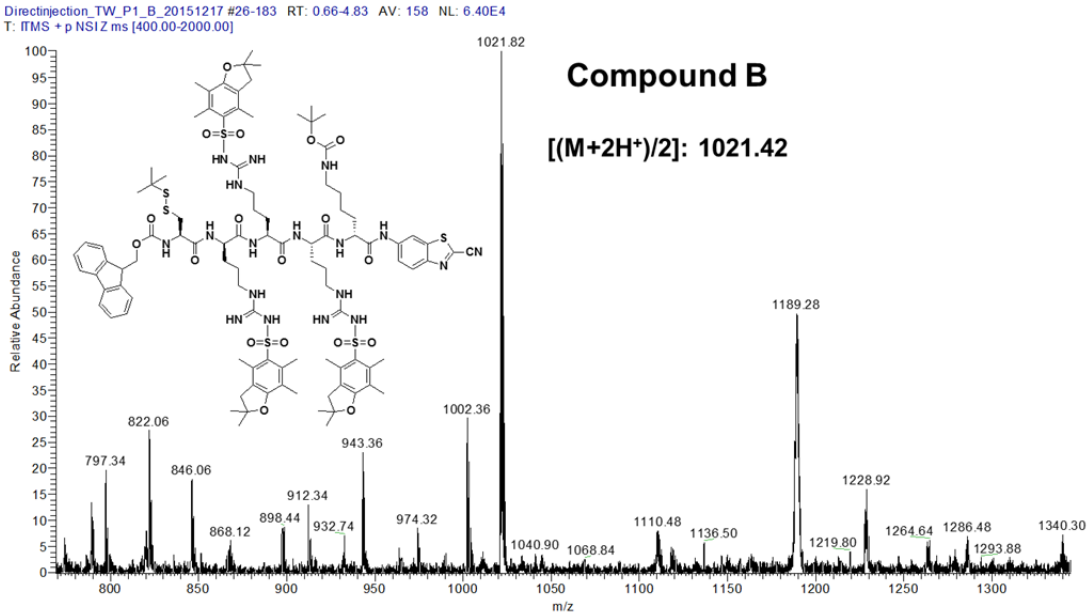


Figure S1. ESI-MS spectrum of compound **B**.

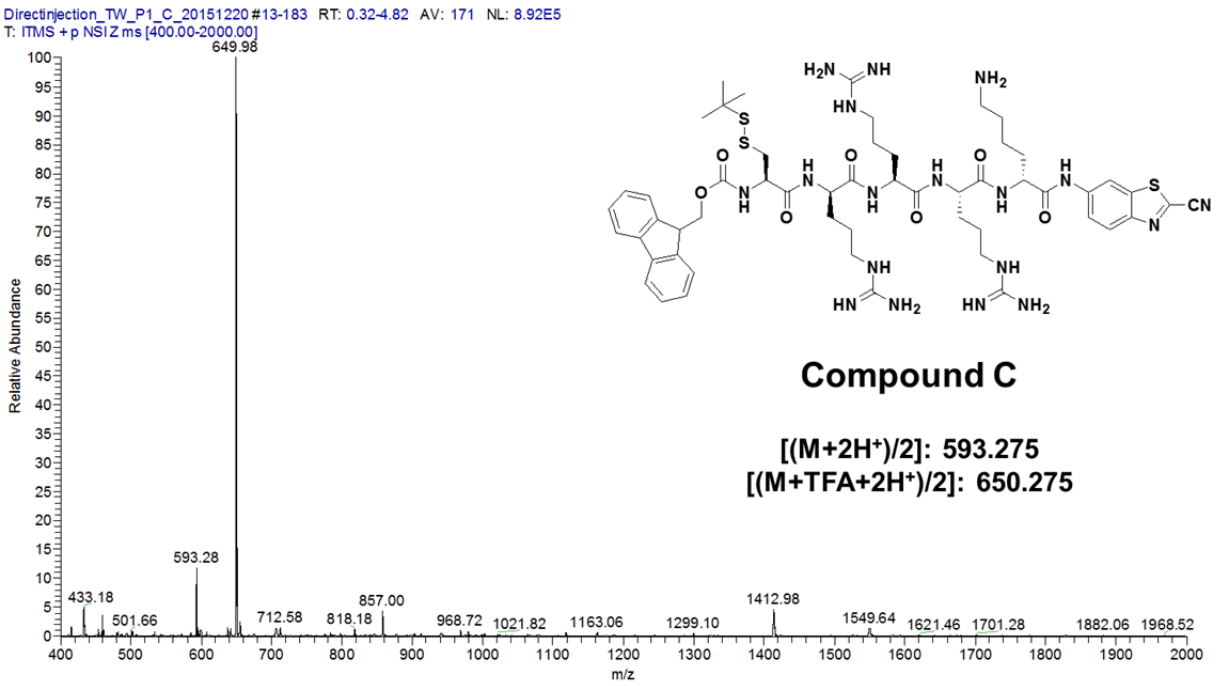


Figure S2. ESI-MS spectrum of compound **C**.

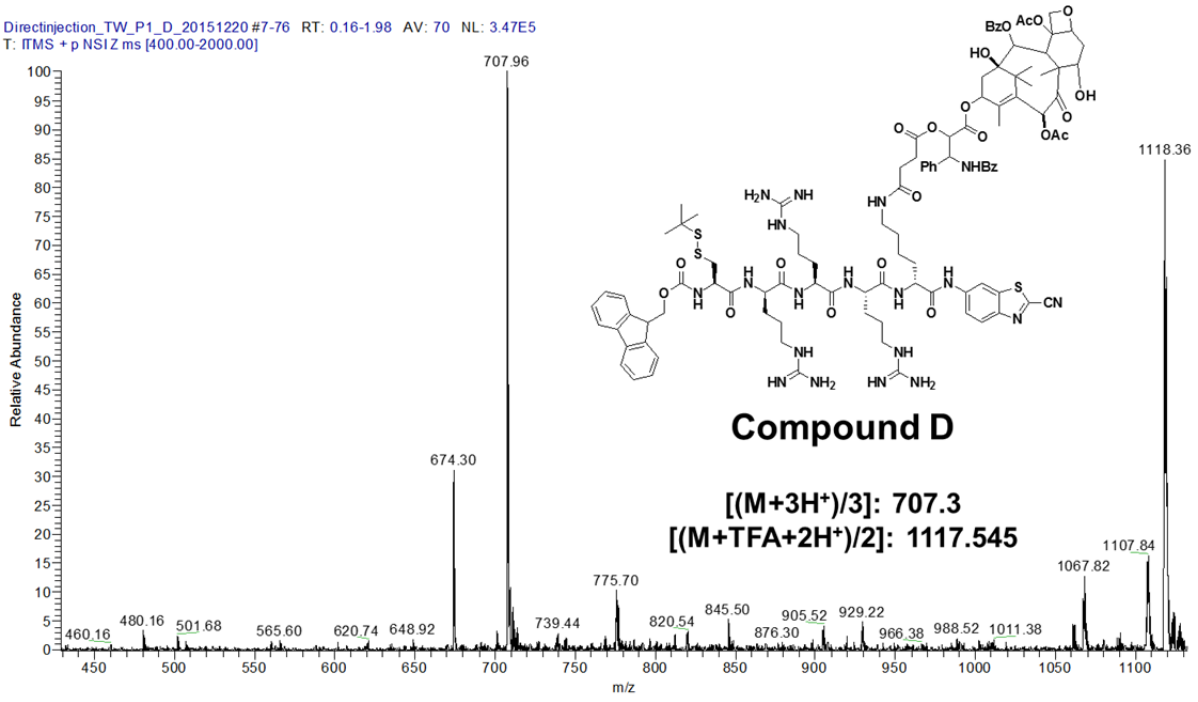


Figure S3. ESI-MS spectrum of compound **D**.

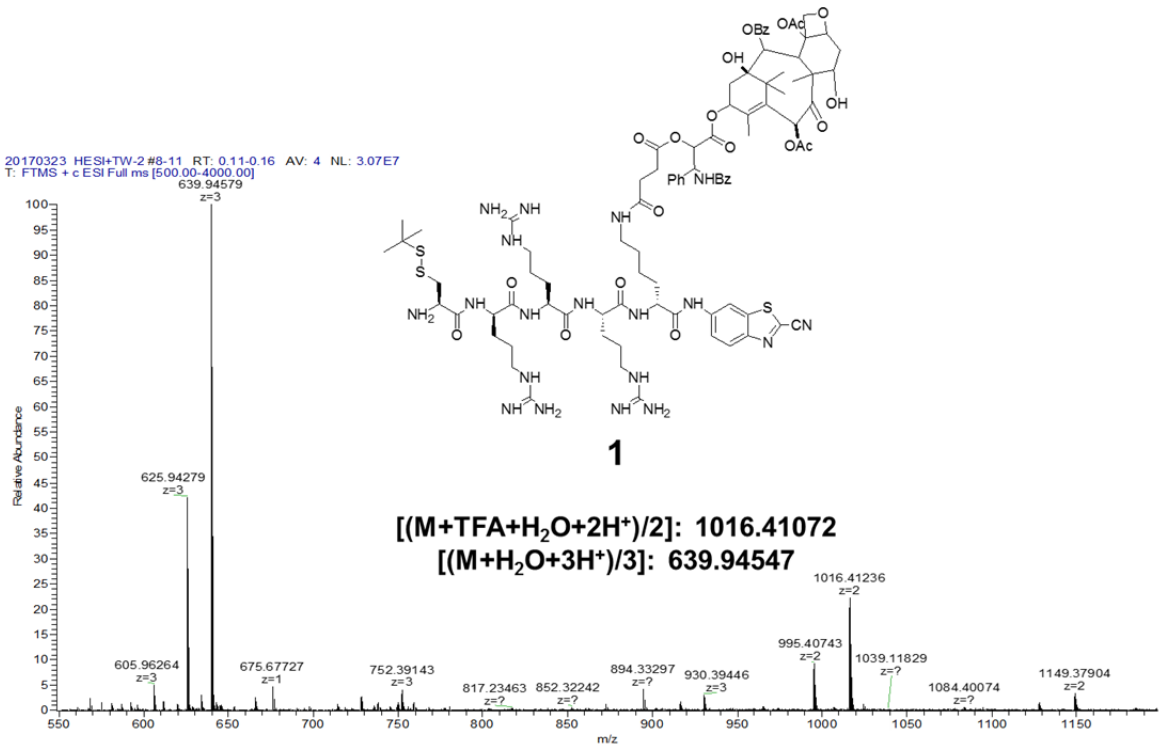


Figure S4. HR-ESI/MS spectrum of **1**.

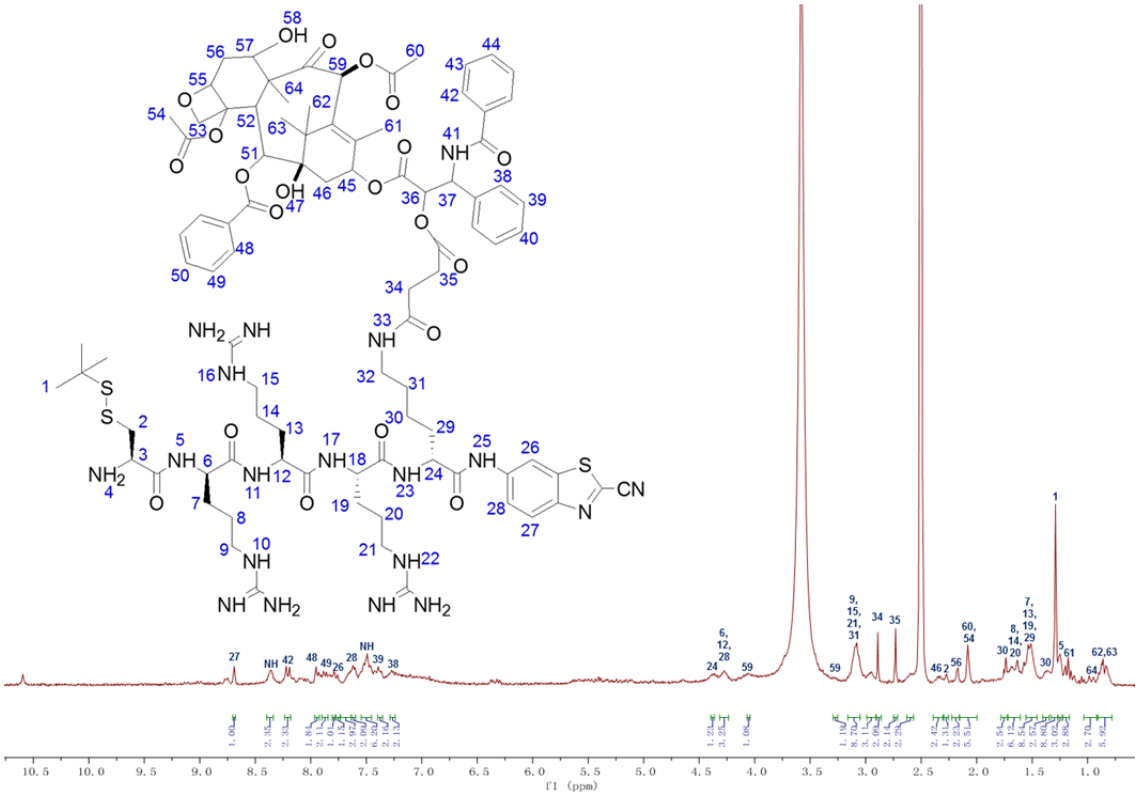


Figure S5. ^{1}H NMR spectrum of **1** in d_6-DMSO.

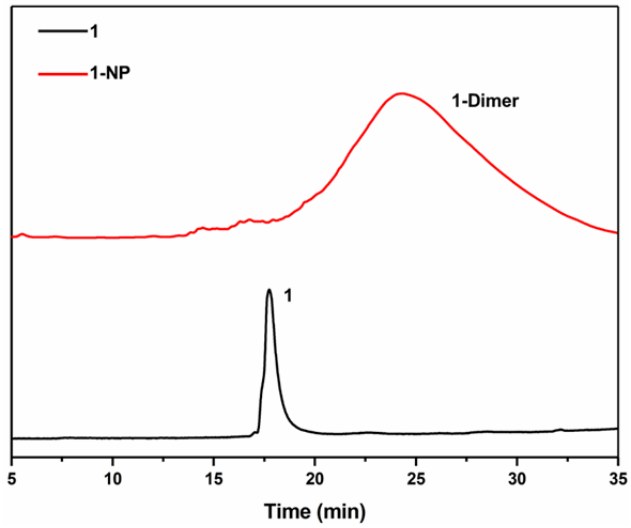


Figure S6. HPLC traces of **1** (black), and **1-NP** dispersion (red) at 320nm.

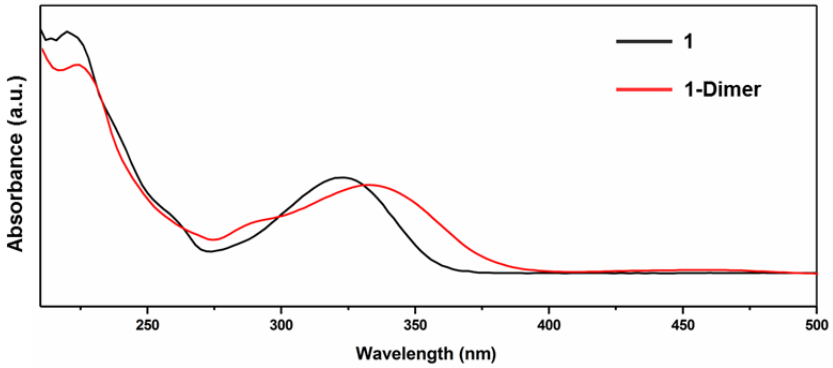


Figure S7. UV-vis spectra of **1** (black) and **1-Dimer** (red) in Figure S6.

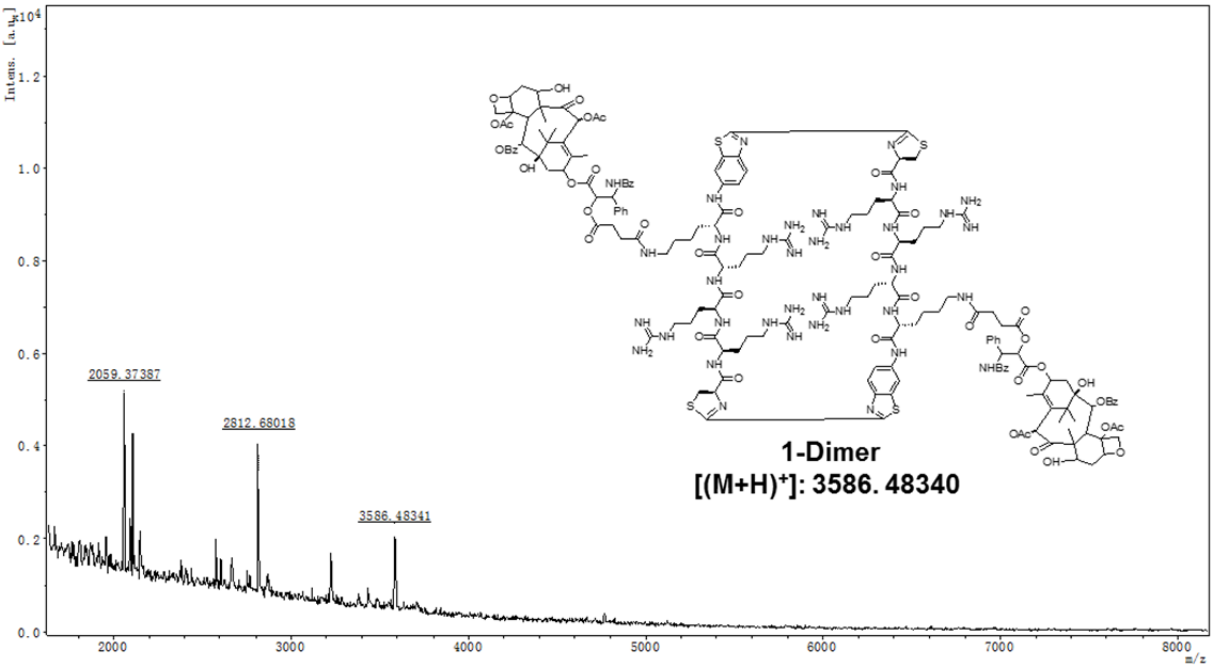


Figure S8. HR-MALDI-TOF/MS spectrum of **1-Dimer** in Figure S6.

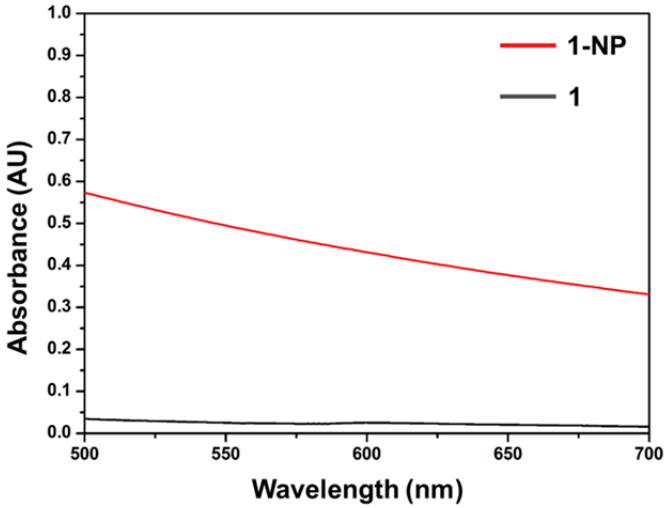


Figure S9. Absorption spectra (500-700 nm due to the light scattering) of **1** and **1-NP** (100 μM) in PBS (black), incubated with TCEP (1 mM) in PBS at pH 7.4 (red), respectively.

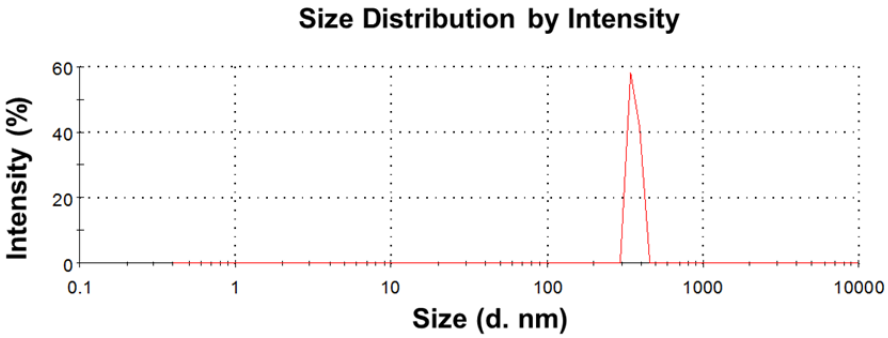


Figure S10. Dynamic light scattering (DLS) measurements of **1** (100 μM) treated with TCEP (1 mM) in PBS at pH 7.4.

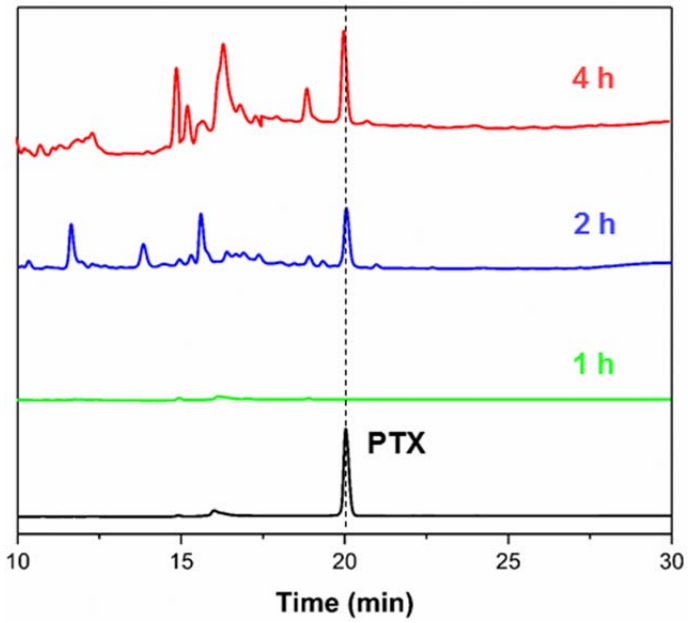


Figure S11. HPLC traces of **PTX** (black), supernatant of **1-NP** after incubation with *Thermomyces lanuginosus* lipase at 1000 U/mL for 1 h (green), 2 h (blue), or 4 h (red). Wavelength for detection: 254 nm.

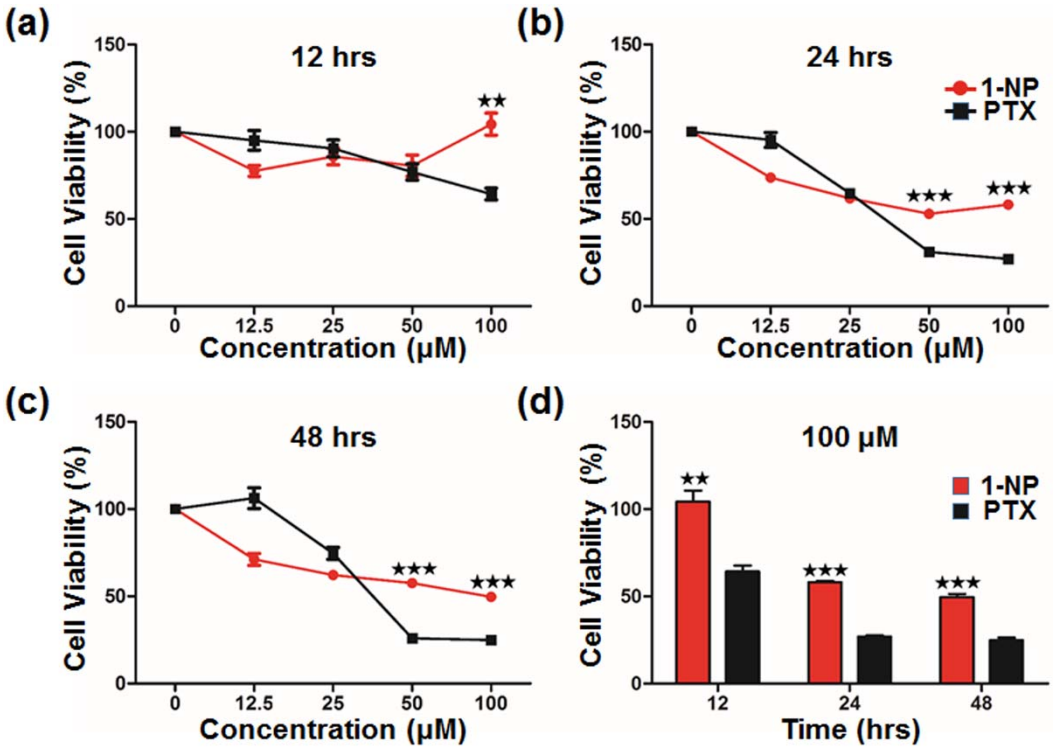


Figure S12. Cell viability study of BMDMs incubated with **1-NP** or **PTX**. BMDMs were incubated with **1-NP** or **PTX** at indicated concentrations for 12 h (a), 24 h (b), or 48 h (c), respectively. (d) Cell viability comparison between the BMDMs incubated with **1-NP** or **PTX** at 100 μM at different time points. Error bars indicate the standard error of mean (SEM). **p < 0.01, ***p < 0.001.

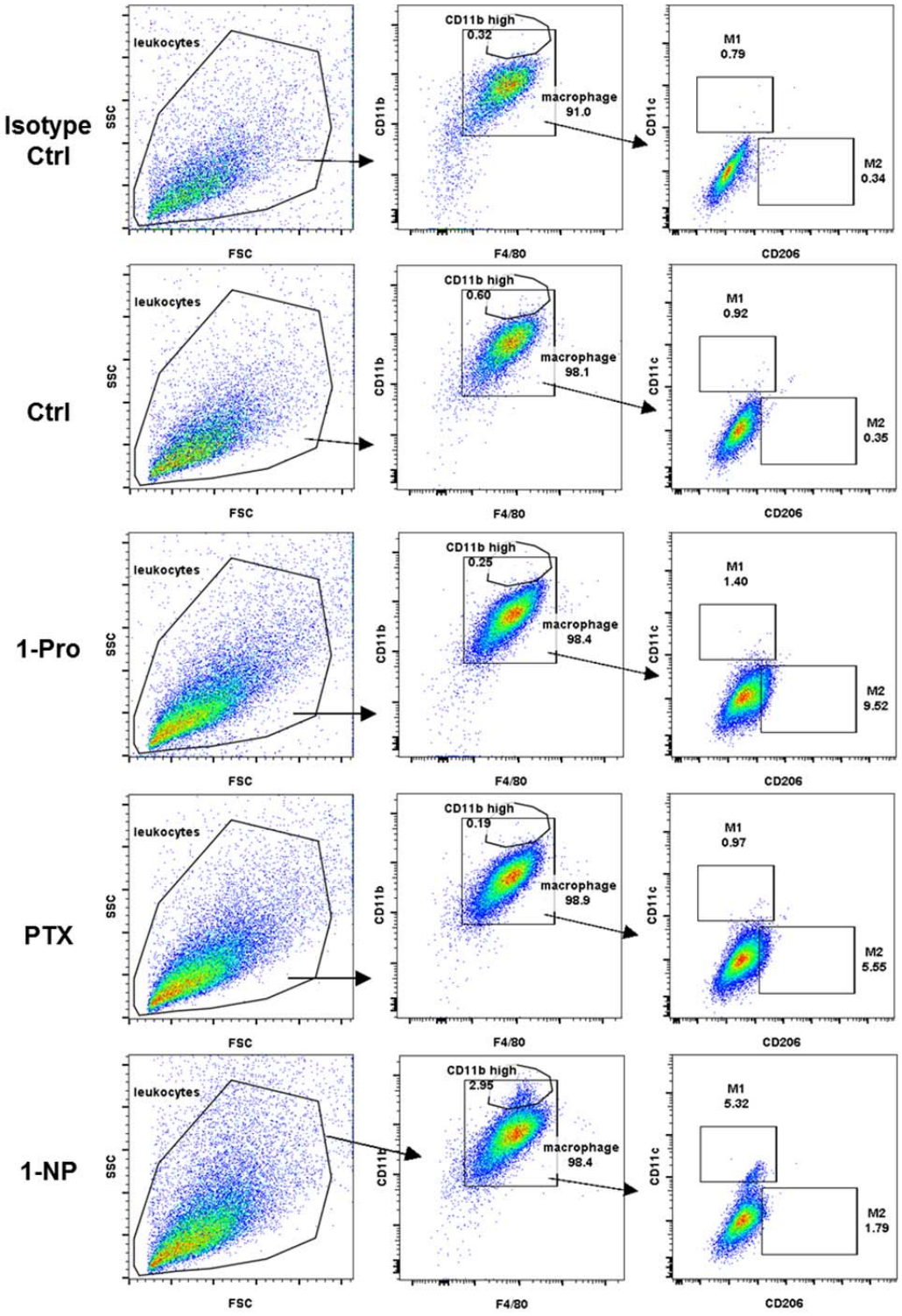


Figure S13. The example gating strategy of BMDMs in co-incubating experiments. Here is the gating way for M0 group, and the other two groups take similar strategies.

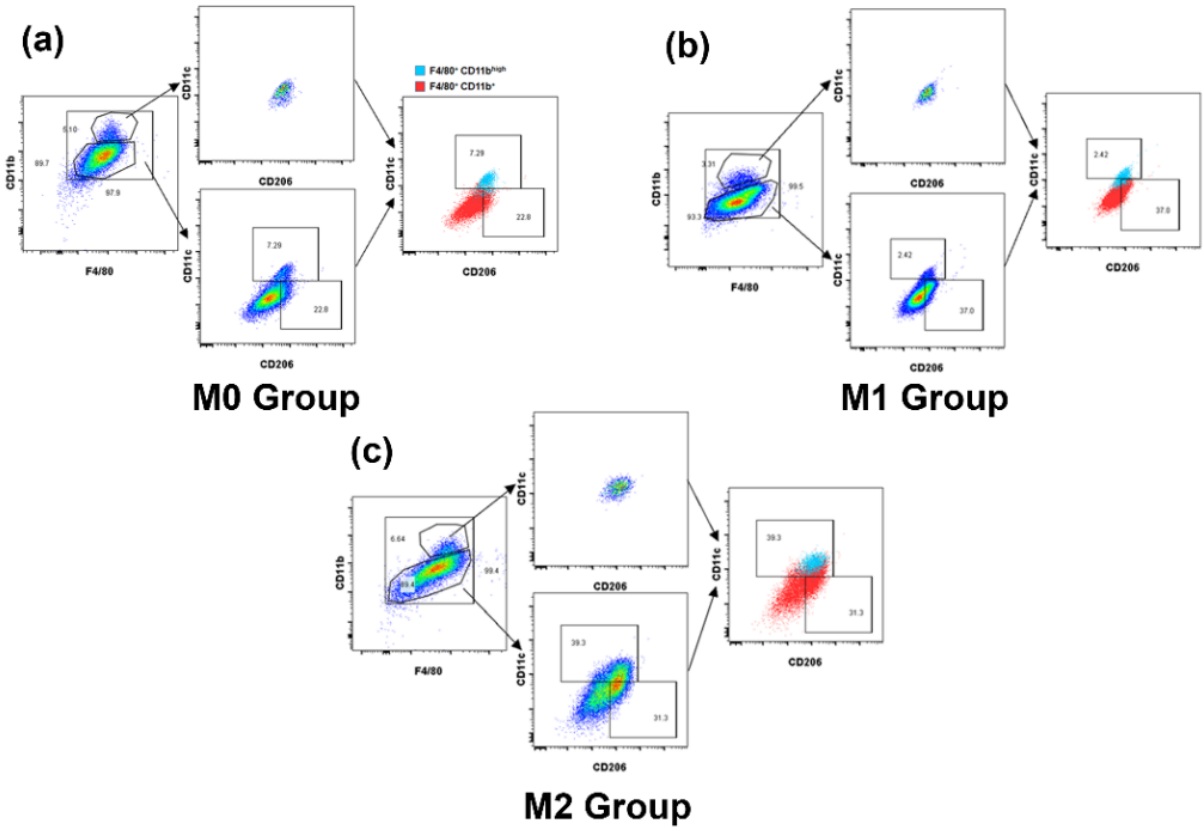


Figure S14. Analysis of BMDMs incubated with **1-NP** in different groups. The subset of $F4/80^{+}$ $CD11b^{high}$ cells obtains the same expression levels of CD11c and CD206 with the $F4/80^{+}$ $CD11b^{+}$ $CD11c^{+}$ cells.

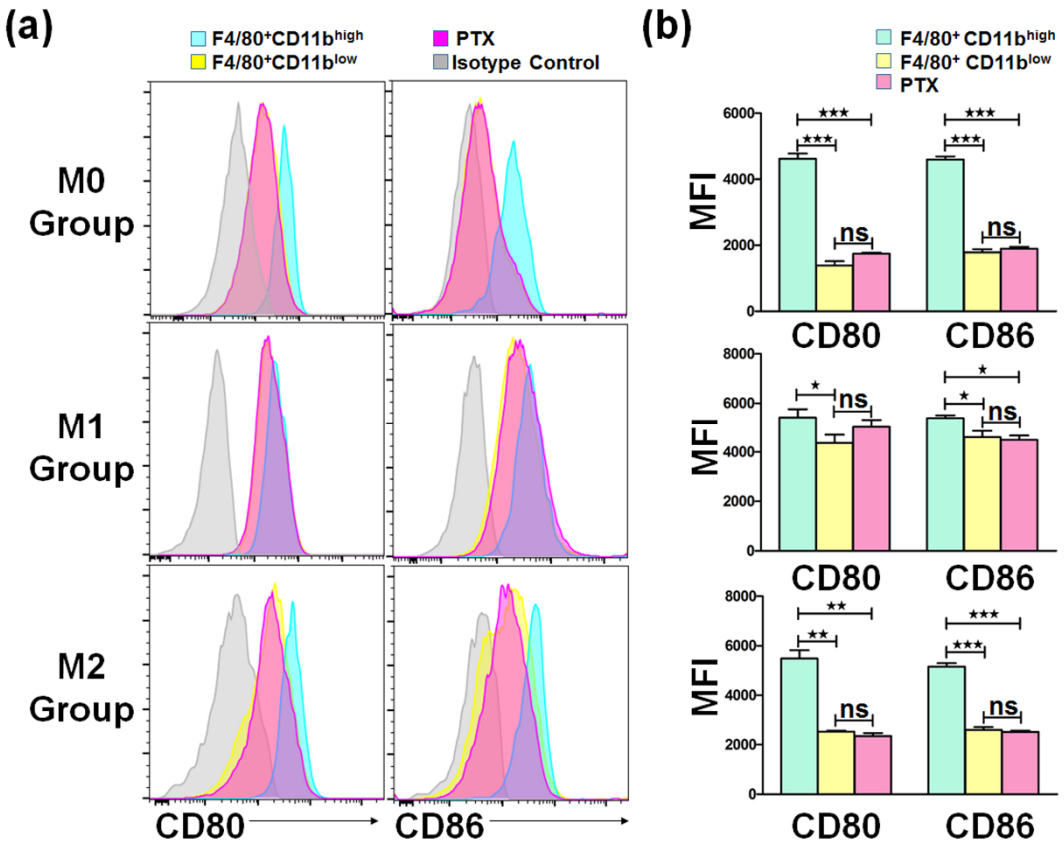


Figure S15. **1-NP** stimulated F4/80^{+} CD11b^{high} BMDMs to upregulate the expression levels of CD80 and CD86. (a) Representative flow cytometric profiles of CD80 and CD86 expression on the gated population of F4/80^{+} CD11b^{high} (green) and F4/80^{+} CD11b^{low} (yellow) of BMDMs incubated with **1-NP**, and F4/80^{+} CD11b^{+} (purple) of BMDMs incubated with **PTX**. (b) The expression levels of surface molecules presented in (a). The results are illustrated as the mean ± SEM of the mean fluorescence intensity (MFI) for each group. The values for F4/80^{+} CD11b^{high} cells are shown in green, the values for F4/80^{+} CD11b^{low} are shown in yellow, and the values for F4/80^{+} CD11b^{+} cells of **PTX** co-incubating BMDMs are shown in purple. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; $p > 0.05$, not significant, ns.

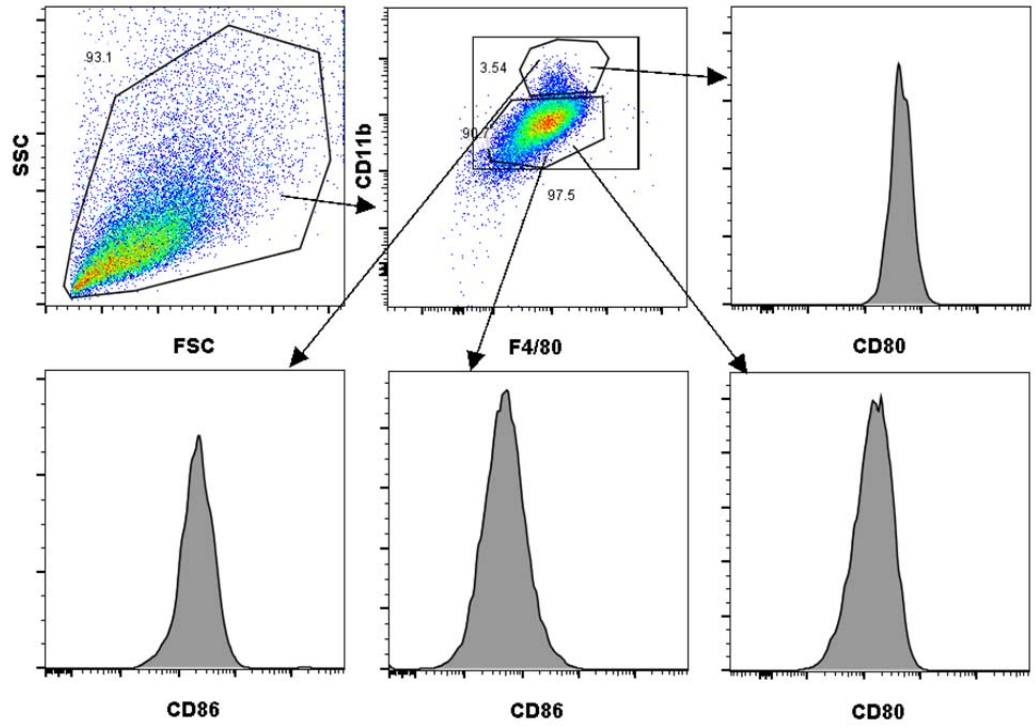


Figure S16. The example gating strategy for analysis of the co-stimulatory molecules on BMDMs incubated with **1-NP** or **PTX**.

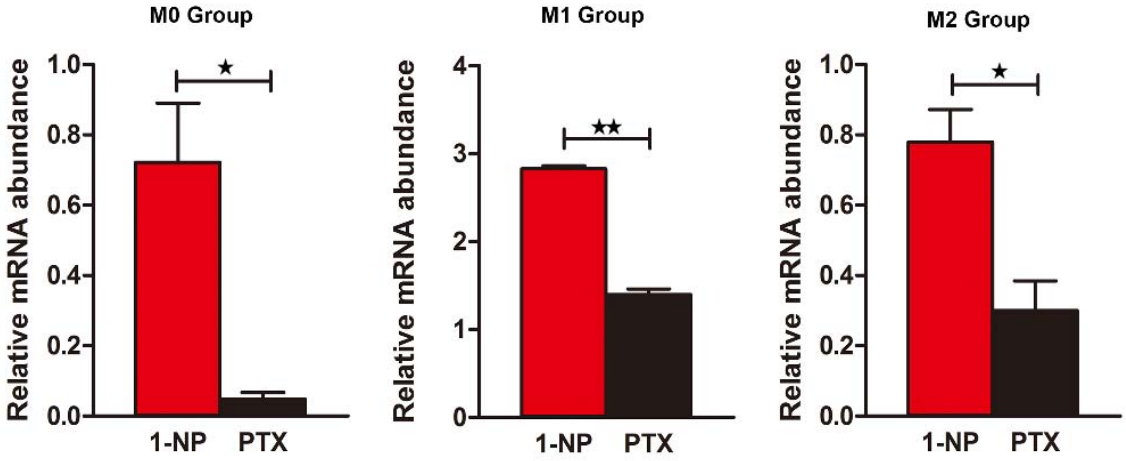


Figure S17. RT-PCR detects the gene expression levels of IL-1β by BMDMs from different groups. Error bars indicate the standard error of mean (SEM), *p < 0.05, **p < 0.01.

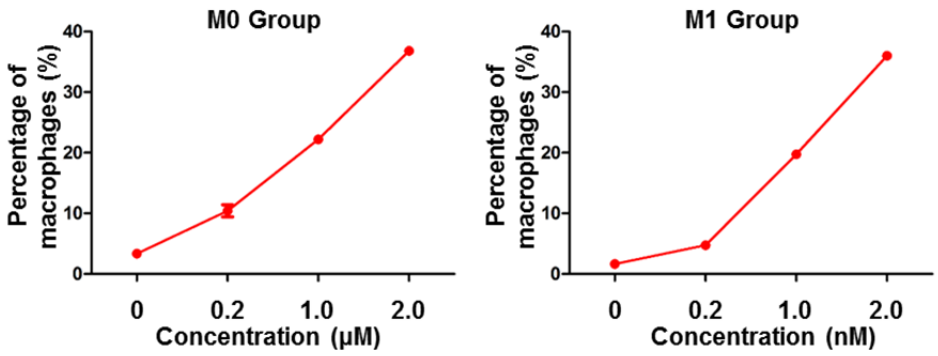


Figure S18. Statistical curves stand for the percentages of F4/80^{+} CD11b^{+} CD11c^{+} cells of BMDMs in M0 and M1 groups, which were co-incubated with concentration gradient of **1-NP** (equivalent to 0, 0.2, 1.0, and 2.0 μM **PTX**).

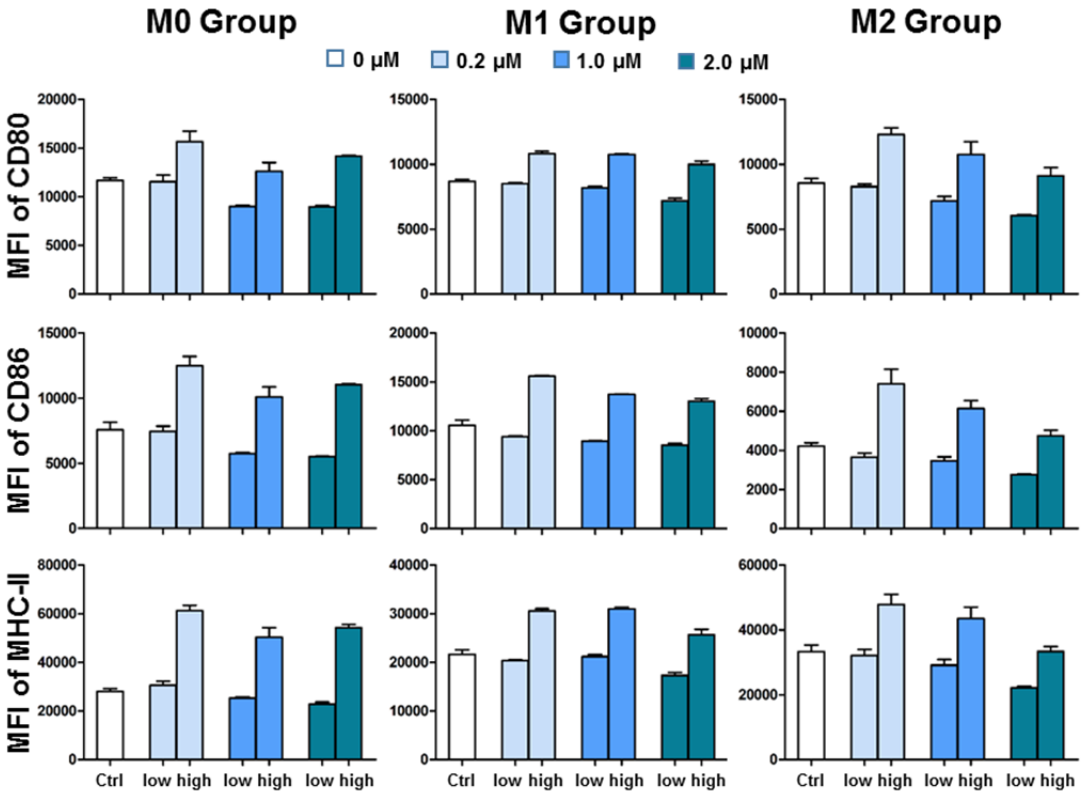


Figure S19. Bar graphs present the MFI of CD80, CD86, and MHC-II on BMDMs with a concentration gradient of **1-NP** (equivalent to 0, 0.2, 1.0, and 2.0 μM **PTX)** co-incubation in different groups. Herein, "low" stands for F4/80^{+} CD11b^{low} cells and "high" stands for F4/80^{+} CD11b^{high} cells.

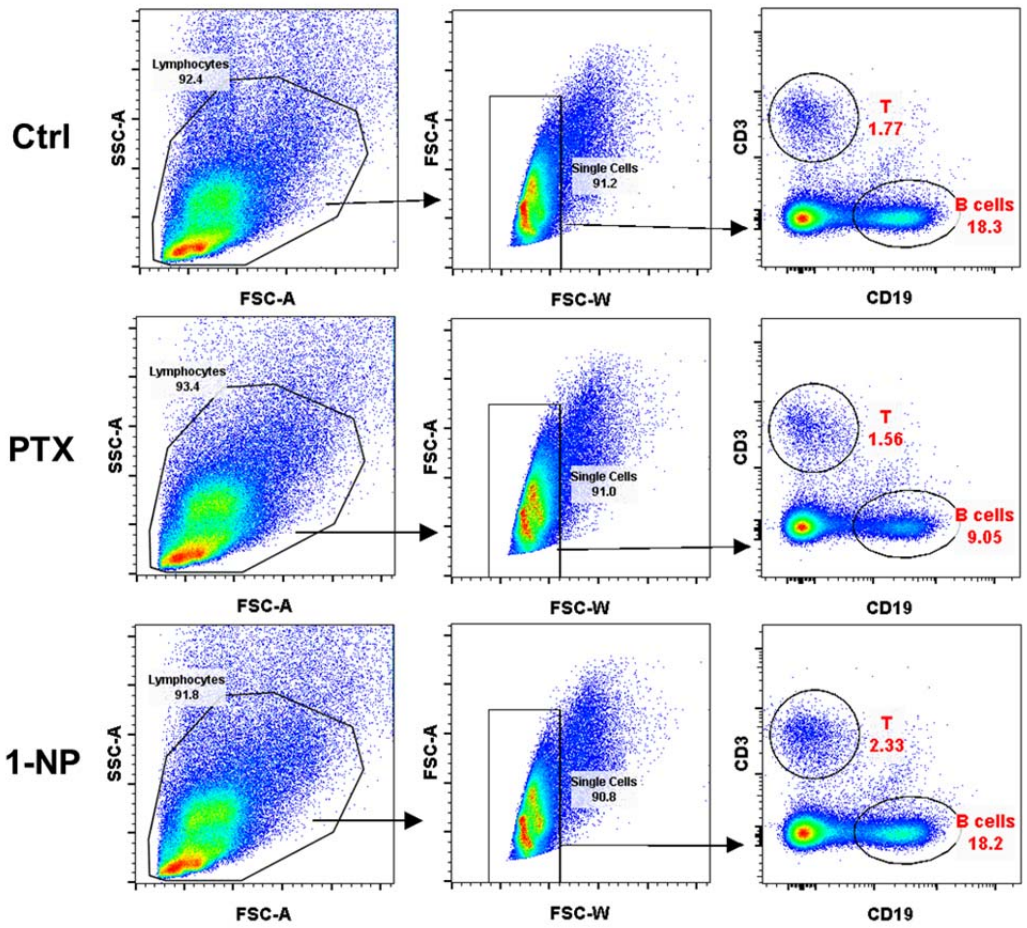


Figure S20. Representative flow cytometric gating strategy of lymphocyte subsets shown in Figure 5 and 6. Here is the bone marrow basic subsets gating strategy, which shows the $CD19^{+}$ B cells percentages decreased significantly more in **PTX** group than Ctrl or **1-NP** group.

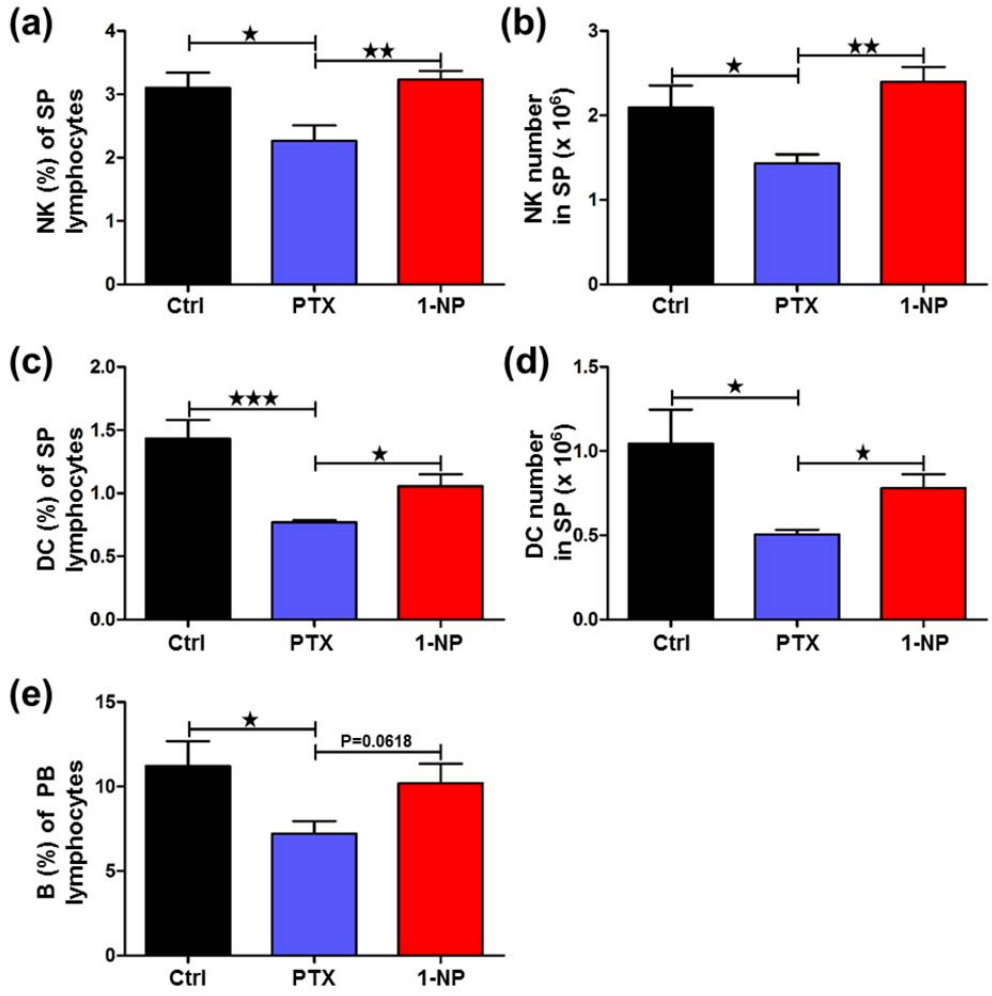


Figure S21. Bar graphs presenting the percentages of spleen (SP) NK (a) and DC (c) in Ctrl, **PTX**, and **1-NP** groups. (b) and (d) are matching graphs of the absolute numbers of these cell subsets in (a) and (c), respectively. (e) Bar graph of the percentages of B cells in periphery blood (PB). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

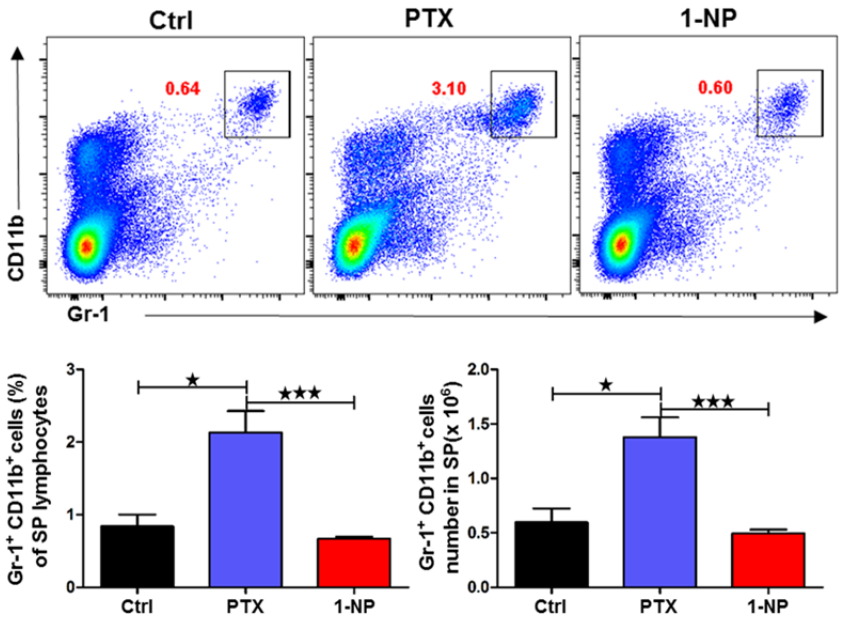


Figure S22. Top, the flow cytometric profiles of Gr-1$^+$ CD11b$^+$ MDSCs gated from spleen leukocytes in different groups. Bottom, bar graphs present the percentages (left) and absolute numbers (right) of Gr-1$^+$ CD11b$^+$ MDSCs in spleens of different experimental groups. *p < 0.05, ***p < 0.001.

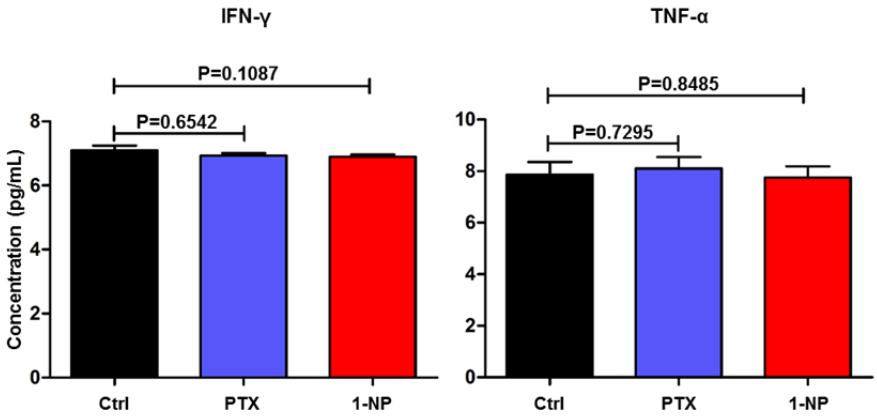


Figure S23. Cytometric Bead Array (CBA) assay of serum inflammation cytokine levels in treated mice. B6 tumor-bearing mice were divided into Ctrl，**PTX**, and **1-NP** groups, injected with PBS, **PTX**, or **1-NP** dispersion daily for 12 days, respectively. At day 13, blood serum was collected and inflammation cytokines were measured. P > 0.05, not significant.

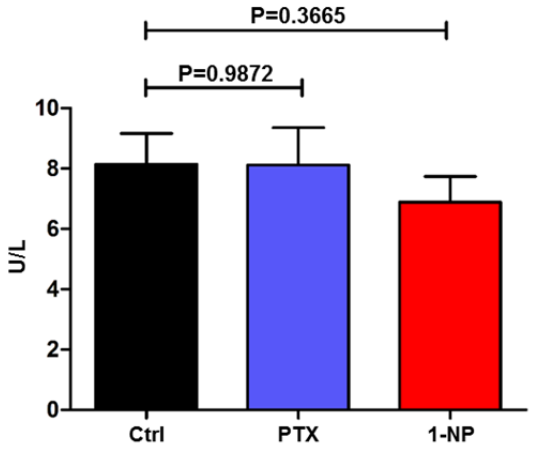


Figure S24. Serum alanine aminotransferase (ALT) levels in treated mice. B6 tumor-bearing mice were divided into Ctrl，**PTX**, and **1-NP** groups, injected with PBS, **PTX**, or **1-NP** dispersion daily for 12 days, respectively. At day 13, blood serums were collected and ALT levels were measured. P > 0.05, not significant.

Table S1. Primers used in real time-qPCR.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
Tnf	AAGCCTGTAGCCCACGTCGTA	AGGTACAACCCATCGGCTGG
Il1b	TCCAGGATGAGGACATGAGCAC	GAACGTCACACACCAGCAGGTTA
Ccr2	ATCCACGGCATACTATCAACATC	CAAGGCTCACCATCATCGTAG
Ccr7	TGTACGAGTCGGTGTGCTTC	GGTAGGTATCCGTCATGGTCTTG
inos	ACATCGACCCGTCCACAGTAT	CAGAGGGGTAGGCTTGTCTC
Gapdh	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

Primer sequences were retrieved from the online PrimerBank database

Table S2. HPLC condition for the purification of **1**, **1-Pro**, **PTX**, and **1-NP**.

Time (minute)	Flow (mL/min.)	H_2O % (0.1%TFA)	CH_3CN % (0.1%TFA)
0	3.0	80	20
3	3.0	80	20
35	3.0	20	80
37	3.0	20	80
38	3.0	80	20
40	3.0	80	20

4. References

1. X. Q. Wang, W. Jiang, Y. Q. Yan, T. Gong, J. H. Han, Z. G. Tian and R. B. Zhou, *Nat. Immunol.*, 2014, **15**, 1126-1133.
2. Q. Jiang, H. Wei and Z. Tian, *J. Immunother*, 2008, **31**, 555-562.